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Geobacteraceae Community Composition Is Related to Hydrochemistry and Biodegradation in an Iron-Reducing Aquifer Polluted by a Neighboring Landfill†

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Relationships between community composition of the iron-reducing *Geobacteraceae*, pollution levels, and the occurrence of biodegradation were established for an iron-reducing aquifer polluted with landfill leachate by using cultivation-independent *Geobacteraceae* 16S rRNA gene-targeting techniques. Numerical analysis of denaturing gradient gel electrophoresis (DGGE) profiles and sequencing revealed a high *Geobacteraceae* diversity and showed that community composition within the leachate plume differed considerably from that of the unpolluted aquifer. This suggests that pollution has selected for specific species out of a large pool of *Geobacteraceae*. DGGE profiles of polluted groundwater taken near the landfill (6- to 39-m distance) clustered together. DGGE profiles from less-polluted groundwater taken further downstream did not fall in the same cluster. Several individual DGGE bands were indicative of either the redox process or the level of pollution. This included a pollution-indicative band that dominated the DGGE profiles from groundwater samples taken close to the landfill (6 to 39 m distance). The clustering of these profiles and the dominance by a single DGGE band corresponded to the part of the aquifer where organic micropollutants and reactive dissolved organic matter were attenuated at relatively high rates.

Iron is one of the most abundant elements in the earth crust, and in many subsurface environments, its reduction is the predominant microbial redox process (16). Because the degradation of organic compounds leads to the rapid depletion of oxygen and nitrate, iron reduction frequently becomes dominant after pollution with organic matter (3, 16).

Iron reduction is also the major redox process in many landfill leachate-polluted aquifers (4, 30). In the past, landfills were not lined, and leachate could contaminate aquifers with a complex mixture of organic and inorganic compounds. Natural attenuation of organic compounds in leachate-polluted groundwater, including those of toxic aromatic compounds like toluene and benzene, is especially observed under iron-reducing conditions (4, 30).

Natural attenuation under iron-reducing conditions also occurs in the aquifer underlying the Banisveld landfill, The Netherlands (22, 30). Molecular fingerprints of *Bacteria* and *Archaea* communities have been found to be related to the presence of pollution and the type of redox process at this location, but no such correlations were observed with biodegradation of dissolved organic carbon (DOC) or benzene, toluene, ethylbenzene, xylene, and naphthalene (22). Members of the family *Geobacteraceae* accounted for a considerable proportion of the microbial community in the polluted aquifer, up to 25% of bacterial counts (22). Enrichment of *Geobacteraceae* generally occurs upon the stimulation of dissimilatory metal reduction by the introduction of organic electron donors into aquifer sediments (2, 10, 11, 20, 25). *Geobacter metallireducens* and *Geobacter grbiciae* are the only iron-reducing species described to date that are capable of aromatic hydrocarbon degradation (5, 14), while *Geobacter* spp. were implicated in anaerobic benzene degradation (23). Therefore, we proposed that *Geobacteraceae* are responsible for much of the biodegradation of organic compounds in landfill leachate (22). If so, more detailed knowledge on the diversity and community structure of *Geobacteraceae* will improve insight into the link between microbial community composition and natural attenuation of landfill leachate. This knowledge will aid in the development of monitoring and bioremediation strategies.

Here, we report the results of cultivation-independent, *Geobacteraceae*-specific molecular analyses on groundwater samples from the aquifer underlying the Banisveld landfill. The community composition and diversity of *Geobacteraceae* are indeed related to the occurrence of degradation processes in, and the hydrochemistry of, the polluted aquifer.

MATERIALS AND METHODS

Site description. The Banisveld landfill is located 5 km southwest of Boxtel, The Netherlands. Household refuse and illegal waste were discarded in a 6-m-deep sand pit between 1965 and 1977. In June 1998, a transect of 11 bailer drillings was installed along the direction of groundwater flow (Fig. 1). Each borehole had two or three polyvinyl chloride piezometers, usually one screen above (Fig. 1, labeled "a"), one inside (b), and one below (c) the leachate plume. Samples from piezometer screens were designated by using the distance downstream of the landfill and the position of the screen; e.g., sample 39b is a sample

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† Unfortunately, Henk van Verseveld died 11 July 2003. In remembrance of a remarkable mentor and fine colleague.

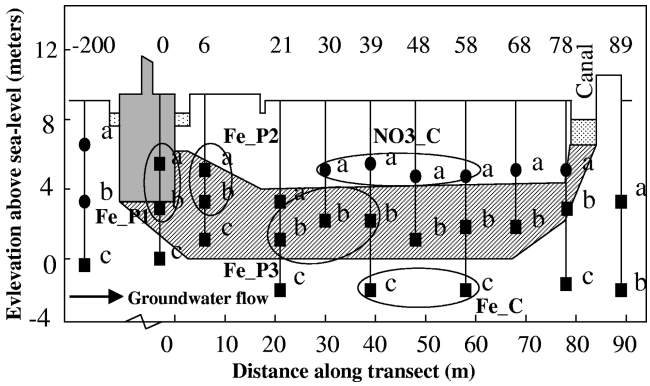

FIG. 1. Cross-section of the Banisveld landfill (shaded area) and the plume of leachate (hatched area) downstream of the landfill, demonstrating the locations of the 11 boreholes. Each borehole is indicated by a number corresponding to the distance (in meters) from the downstream border of the landfill. Two to three screens were placed per borehole, indicated by a character (a, b, or c) and the following symbols: ●, screen from which a groundwater sample with a nitrate concentration of >0.5 mg/liter was withdrawn in September 1998; ■, no nitrate present. The five oval circles and their codes refer to Table 1. DNA extracts from groundwater samples taken from screens within the oval circles were pooled and used for the construction of *Geobacteraceae* clone libraries. Additional characteristics of the research site are given in Materials and Methods.

from the plume, 39 m downstream. Extensive hydrochemical characterization was performed in 1998 and 1999 (30). Within the plume, concentrations of the dissolved organic matter, naphthalene (N), and the aromatic micropollutants benzene (B), ethylbenzene (E), and xylene (X) decrease, with naphthalene, ethylbenzene, and xylene disappearing within the first 21 m (30). Other micropollutants, such as chlorinated hydrocarbons, were never detected (gas chromatography-flame ionization detector/mass spectrometry detection limit of 0.2 μg/liter). The micropollutants (maximum, 221 μg/liter) formed a small fraction of the DOC (62 to 110 mg/liter) in the plume of pollution (30). Reactive transport modeling indicated that DOC consisted of a persistent (67% of DOC) and a reactive (33%) fraction underneath the plume (29). The reactive fraction was degraded with a first-order rate constant of 1.06×10^{-1} year^{-1} and was nearly completely consumed in the first 39 m downstream of the landfill. The persistent fraction was degraded much slower, with a first-order rate constant of 1.03×10^{-2} year^{-1}. Only 10% of the persistent DOC was degraded in the first 39 m downstream of the landfill. As inferred from a combination of hydrogen-gas measurements, analysis of redox species [oxygen, nitrate, Fe(II), sulfide, sulfate, and methane], thermodynamic calculations, and inverse geochemical modeling, iron reduction was the dominant redox process inside and beneath the plume, while nitrate reduction was observed above the plume (Fig. 1) (30). Nitrate reduction above the plume was also indicated by both an enriched $\delta^{15}NO_3$ and partial N_2 pressure exceeding atmospheric equilibrium (30). The maximum rate of iron reduction determined by reactive transport modeling was 1.5×10^{-3} mol/liter/year below the landfill and decreased with distance from the landfill (29).

***Geobacteraceae*-specific denaturing gradient gel electrophoresis (DGGE) profiling and data analysis.** The same DNA extracts previously used to profile *Bacteria* and *Archaea* communities were used in this study (22). These DNA extracts were isolated from groundwater sampled in September 1998 and stored at −20°C for 3 years before the work described in this paper was started.

To profile *Geobacteraceae* communities in DGGE, a nested PCR approach was applied. First, a *Geobacteraceae*-specific PCR (25) was performed to amplify a 0.8-kb 16S rRNA gene fragment in a total volume of 25 μl containing 0.4 μM primer 8f (8), 0.4 μM primer 825r (25), 0.4 mM deoxynucleoside triphosphates, 10 μg bovine serum albumin, Expand buffer, and 2.6 U Expand enzyme (Boehringer, Mannheim, Germany) as well as 1 μl of undiluted DNA extract. PCR was performed in a Perkin-Elmer DNA Thermo Cycler, and conditions are as follows: 94°C for 4 min and then touchdown primer annealing from 65°C to 56°C (decreasing 1°C per 2 cycles), followed by 15 cycles at an annealing temperature of 55°C, with a final elongation step at 72°C for 5 min. PCR products were purified (Qiaquick Rep purification kit; QIAGEN), and 1 μl of 1/100 diluted PCR product was used for the second round of amplification using *Bacteria*-specific primers in a 25-μl reaction volume containing 0.4 μM primer F357-GC (19), 0.4 μM primer R518 (19), 0.4 μM deoxynucleoside triphosphates, 10 μg bovine serum albumin (Biolabs, United Kingdom), and 2.5 U *Taq* polymerase. Amplification was performed as follows: 94°C for 4 min and then 35 cycles of 94°C for 1 min, 54°C for 1 min, and 72°C for 1 min, with a final elongation phase at 72°C for 5 min.

DGGE was performed with the Bio-Rad DCode system. PCR product was loaded onto 1-mm-thick 8% (wt/vol) polyacrylamide (37.5:1 acrylamide-bisacrylamide) gels containing a 30 to 55% linear denaturing gradient. One hundred percent denaturant is defined as 7 M urea and 40% (vol/vol) formamide. Gels were run in 1× TAE buffer (40 mM Tris-acetate, 1 mM Na-EDTA, pH 8.0) at 200 V for 4 h. Gels were stained in 1× TAE buffer containing 1 μg ml^{-1} ethidium bromide and recorded with a charge-coupled-device camera system (The Imager; Appligen, Illkirch, France).

Gel images were converted, normalized, and analyzed by the GelCompar II software package (Applied Maths, Kortrijk, Belgium). To facilitate the conversion and normalization of gel images, a marker consisting of 12 clones was added. DGGE profiles were compared using a band assignment-independent method (Pearson product-moment correlation coefficient and unweighted-pair group clustering method using arithmetic averages), as well as a method based on band presence/absence (Jaccard coefficient; $S_J = \frac{n_{AB}}{n_A + n_B - n_{AB}}$, in which n_A, n_B, and n_{AB} are the total number of bands in track A and in track B and the number of bands common to tracks A and B, respectively). The Pearson product-moment correlation coefficient analysis is affected much less than band-based similarity coefficients by the amount of PCR products loaded onto gel and is a fast, objective method to compare microbial community profiles (32). In band assignment, a 1% band position tolerance (relative to the total length of the gel) was applied, which indicates the maximal shift allowed for two bands in different DGGE tracks to be considered as identical. Band presence or absence in DGGE tracks was scored as 1 or 0, respectively. These 1/0 numbers were exported to the spreadsheet program Excel. To determine whether a certain DGGE band was specific for a certain condition, statistical analyses on 2 by 2 tables containing the number of times the particular band was absent or present for a certain condition (clean versus polluted, nitrate reducing versus iron reducing) were conducted by Fisher's exact test using Systat 7.0 (SPSS Inc).

Phylogenetic analysis of *Geobacteraceae* 16S rRNA genes. Five clone libraries were constructed. Each library corresponded to a combination of a particular pollution level (P, polluted; C, clean) and redox process (NO_3, nitrate reducing; Fe, iron reducing) and were coded Fe_P1, Fe_P2, Fe_P3, Fe_C, and NO_3_C, (Fig. 1 and Table 1). The clone libraries were constructed from composite samples obtained by mixing equal amounts of isolated DNA from the relevant groundwater samples (Table 1). A *Geobacteraceae*-specific PCR with primers 8f and 825r was performed as described above. PCR products were cleaned with the Qiaquick Rep purification kit (QIAGEN, Germany) and cloned into *Escherichia coli* JM109 via the pGEM-T vector system (Promega, Madison, Wis.). Clones were screened by PCR with pGEM-T-specific primers T7 and Sp6. PCR products from transformants with correctly sized insert DNA were used as templates in a PCR with *Bacteria*-specific primers F357-GC and R518 to compare the migration position in DGGE to the DGGE pattern of the environmental sample from which the clone had been derived. Clones were classified into DGGE types based on differences in migration behavior in DGGE. At least one representative clone per type was sequenced. Sequencing PCR was carried out with the ABI PRISM Dye Terminator Cycle Sequencing Core kit (Perkin-Elmer), and the purified products were run on a SEQUAGEL-6 sequence gel (National Diagnostics) in a 373A/DNA Sequencer (Applied Biosystems). Both strands of the 16S rRNA gene were sequenced from *E. coli* positions 8 to 825. Sequences were compared to sequences deposited in the GenBank DNA database by using the BLAST algorithm to obtain the most closely related sequences (1). Chimera checks of the 16S rRNA gene sequences of clones were performed via the Chimera-Check program from RDP (18) and by comparing phylogenetic trees based on the first 400 bp to those based on *E. coli* positions 401 to 825. Chimeric sequences were excluded from further phylogenetic analysis. Sequence alignment was performed by ClustalW and then corrected manually. Distance analysis on unambiguously aligned sequences using the correction of Jukes and Cantor (12) and bootstrap resampling (100 times) were done with the TREECON package (31), and the distance matrix was used to construct a tree via the neighbor-joining method (24).

Molecular detection of *Anaeromyxobacter*, *Geothrix*, and *Shewanella*. *Geothrix*- and *Shewanella*-specific PCRs were carried out as described previously by Snoeyenbos-West et al. (25). *Anaeromyxobacter*-specific PCR was performed according to the method described previously by North et al. (20).

TABLE 1. Codes of the five clone libraries constructed from groundwater samples from the aquifer near the Banisveld landfill[a]

Code	Origin of sample	Pollution	Redox process	No. of clones	No. of *Geobacter* clones (%)	No. of DGGE types	No. of *Geobacter* types (%)
Fe_P1	In and directly underneath landfill	BEXN (221 μg/liter)	Fe(III)	37	32 (86)	13	10 (77)
Fe_P2	Plume, 6 m downstream	BN	Fe(III)	33	25 (76)	15	11 (73)
Fe_P3	Plume, 21–39 m downstream	B	Fe(III)	40	33 (83)	13	9 (69)
Fe_C	Beneath the plume, 39–58 m downstream		Fe(III)	47	30 (64)	17	11 (64)
NO_3_C	Above the plume, 30–58 m downstream		NO_3^-	39	9 (23)	13	2 (15)

[a] Each clone library indicates from which samples it was constructed as well as which type of pollution (B, benzene; E, ethylbenzene; X, xylene; N, naphthalene) was present and which redox process dominated (for more details, see Materials and Methods and reference 30). The samples used to construct the five clone libraries are also indicated in Fig. 1 (ovals). In addition, results of screening of the *Geobacteraceae* clone libraries constructed are listed; the numbers of clones screened per library, the number of confirmed *Geobacteraceae* clones, the number of different banding positions observed in DGGE(DGGE types), and how many of these bands corresponded to *Geobacteraceae* are indicated.

Nucleotide sequence accession numbers. Nucleotide sequences have been deposited in the GenBank database under accession numbers AY752746 to AY752785.

RESULTS

Molecular detection of specific groups of iron reducers. Iron-reducing microorganisms from the genera *Shewanella*, *Geothrix*, *Anaeromyxobacter*, and *Geobacter* are common to various metal-reducing environments. Groundwater samples from the aquifer near the Banisveld landfill were tested for the presence of these microorganisms by using group-specific PCR amplification. Hydrochemical characteristics (redox conditions and the presence of organic pollutants) of these groundwater samples have been described previously (22, 29, 30) and are indicated in Fig. 1 and briefly described in Materials and Methods. *Shewanella* was not detected in any of the composite DNA samples used to generate clone libraries (Table 1), despite the ability of the PCR assay to detect one 16S rRNA gene per amplification reaction (data not shown). After *Geothrix*-specific amplification, low-intensity PCR bands were observed but only for the composite samples Fe_P2, Fe_P3, and NO_3_C. *Anaeromyxobacter* sequences were only detected in composite sample Fe_P1. By contrast, *Geobacteraceae*-specific PCR gave a strong signal for the five composite samples as well as for all 27 individual groundwater sampling locations, indicating that *Geobacteraceae* are widespread in the aquifer. Combined with previous results indicating the dominant contribution of *Geobacter* spp. to microbial communities in the iron-reducing leachate plume (22), the results of these molecular analyses warranted further focus on iron-reducing *Geobacteraceae*.

***Geobacteraceae* community profiling.** *Geobacteraceae* communities in groundwater were investigated by DGGE profiling of *Geobacteraceae*-specific 16S rRNA genes. A large diversity was observed (Fig. 2). In total, 62 different banding positions were detected for the 27 groundwater samples analyzed. The average number of banding positions per groundwater sample was 16, with a minimum of 10 for sample 21c and a maximum of 24 for groundwater sample −200a. There was no significant difference in the average number of bands between groundwater samples stemming from the polluted part and groundwater samples coming from the unpolluted part of the aquifer (analysis of variance, $P > 0.05$).

In order to detect similarities between DGGE fingerprints and to relate these to hydrochemical characteristics, cluster analysis was performed. The analysis was based on the whole densitometric curve of the DGGE profiles and used the Pearson product-moment correlation coefficient (21). Overall, a low similarity was found between samples in terms of their *Geobacteraceae* community profiles, often also when samples from locations with similar redox conditions and pollution levels were compared (Fig. 2). Only samples from the iron-reducing, polluted part close to the landfill (at a distance of 6 to 39 m downstream of the landfill) clearly clustered at a similarity level of 50%; all these fingerprints had an intense band in common (see below). Groundwater samples taken at the same distance from the landfill body but at different depths in the plume were more similar to each other than to samples taken at other distances. The fingerprints from polluted groundwater samples close to the landfill (6 to 39 m downstream) were quite different from the DGGE profiles of the four polluted groundwater samples taken further downstream (48 to 78 m). The latter lacked the aforementioned highly intense band and clustered with samples from unpolluted iron-reducing (58c and 78c) and nitrate-reducing (48a, 58a, 68a, and 78a) groundwater at the same distance from the landfill, at a similarity of 54%. Cluster analysis based on band absence or presence only, i.e., without taking into account band intensity, failed to yield clearly separated groups of samples (less than 30% similarity [data not shown]).

In order to relate the presence of individual DGGE bands to hydrochemical conditions, statistical analysis (Fisher's exact tests) was performed on 2-by-2 tables, containing the number of times a particular band was absent or present for a certain condition (pollution level [clean versus polluted] or redox process [nitrate versus iron reduction]). The eight bands indicated in Fig. 3 were found to be indicative of either pollution (significantly more present in polluted or clean groundwater [$P < 0.05$]) or redox process (significantly more present in groundwater from nitrate- or iron-reducing parts of the aquifer) (Table 2).

A distinctive and dominant band (Fig. 3, band 2) was observed in the DGGE profiles from polluted groundwater samples near the landfill that corresponded to iron-reducing conditions. This band was also observed for polluted groundwater

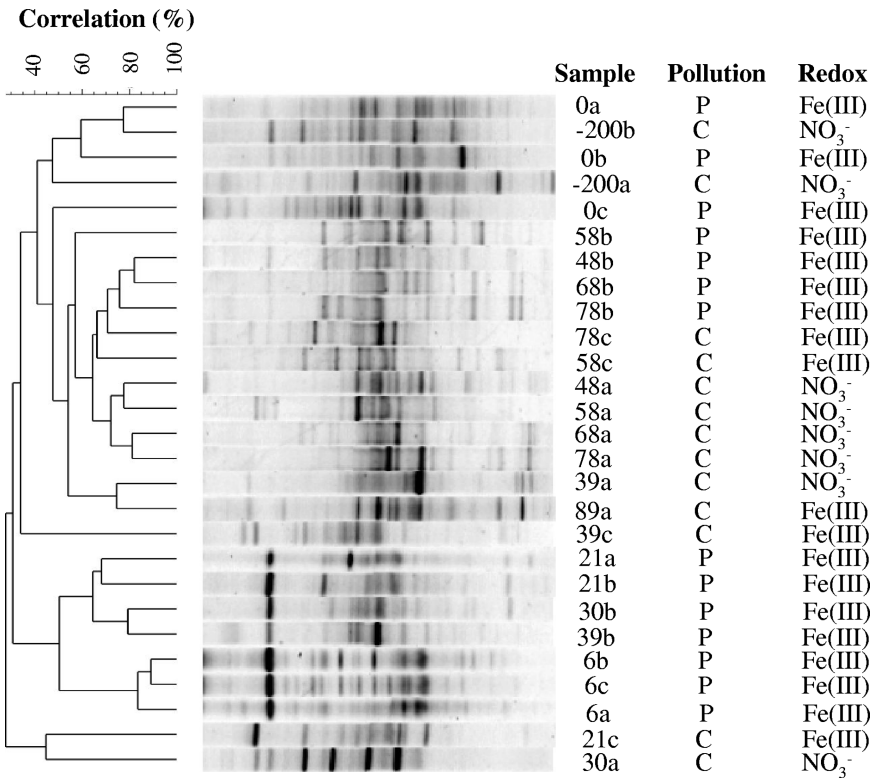

FIG. 2. Unweighted-pair group method using arithmetic averages cluster analysis of DGGE profiles of *Geobacteraceae* (denaturant gradient, 30 to 55%). Sample codes are explained in the legend of Fig. 1. The column marked "pollution" indicates whether the analyzed groundwater sample was polluted (P) or clean (C), and the column "redox" indicates the dominant redox process [Fe(III) for iron reduction and NO_3^- for nitrate reduction].

samples further away from the landfill but at lower intensity (cf. lanes marked 48b to 78b in Fig. 3). It was absent from most clean groundwater samples, with the exception of samples −200b, 30a, 0c, and 78c (Fig. 3). Fisher's exact test revealed that this band was significantly indicative of polluted groundwater ($P = 0.001$) (Table 2). Also, three other, less intense bands (bands 4, 5, and 8) were indicative of polluted groundwater. A plot of the relative intensities of the pollution-indic-

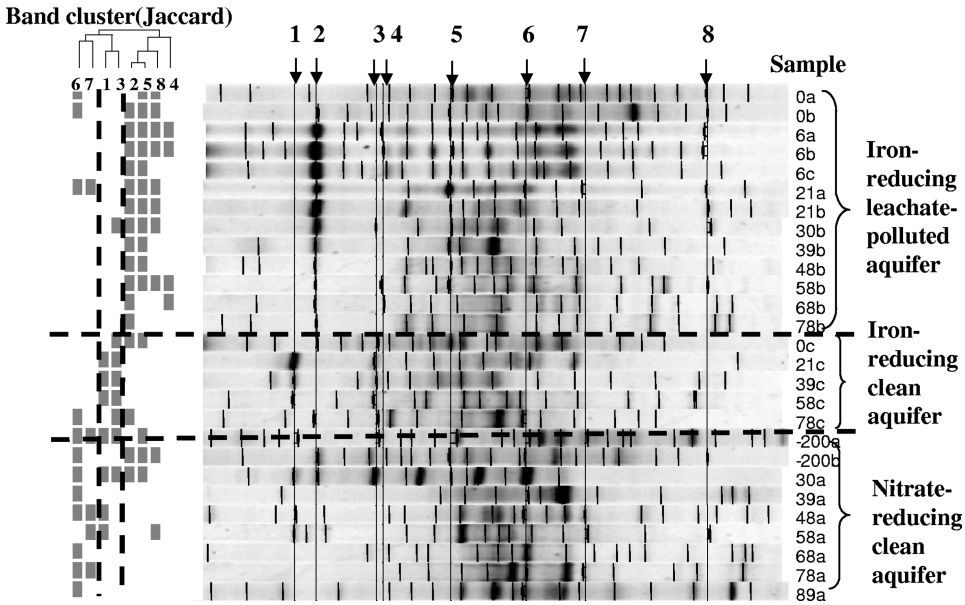

FIG. 3. Band-based analysis of the *Geobacteraceae* DGGE profiles. Numbered arrows indicate bands that are significantly ($P < 0.05$) indicative of pollution level or redox process, as determined by Fisher's exact test (Table 2). On the left is a graphical representation of the occurrence of these indicator bands in the DGGE profiles. Band cluster analysis for this graphical representation was performed using the Jaccard coefficient.

TABLE 2. Results from Fisher's exact tests on the relationship between the presence of individual bands in DGGE profiles and environmental conditions[a]

Polluted vs clean		Iron reduction vs nitrate reduction	
Band position	*P*	Band position	*P*
Band 1	0.006 (clean)	Band 6	0.037 (NO_3)
Band 2	0.001 (polluted)	Band 7	0.030 (NO_3)
Band 3	0.033 (clean)		
Band 4	0.041 (polluted)		
Band 5	0.006 (polluted)		
Band 8	0.018 (polluted)		

[a] DGGE profiles are shown in Fig. 3. Bands that were significantly ($P < 0.05$) indicative of pollution level (clean versus polluted groundwater) are shown on the left, while bands significantly indicative of the dominant redox process (nitrate or iron reduction) are depicted on the right. For each band, the significance (*P* value) is indicated as well as (between parentheses) the specific environmental condition for which it was indicative.

ative bands in the DGGE profiles from plume samples shows that bands 2 and 8 had the highest contribution to DGGE profiles in the first part of the aquifer, i.e., up to 39 m downstream of landfill (Fig. 4A). In this part of the landfill, the concentrations of DOC and aromatic micropollutants also decreased with increasing distance from the landfill (Fig. 4B). In the second part of the plume, more than 39 m downstream of the landfill, the relative intensities of these bands were low, and concentrations of DOC and micropollutants decreased little (Fig. 4).

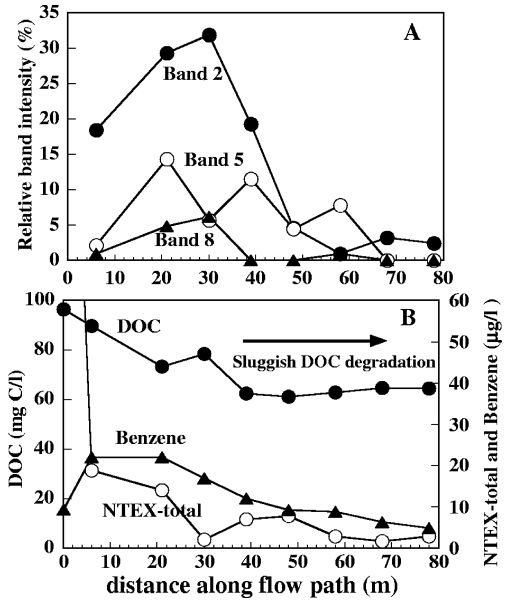

FIG. 4. Relationships between occurrences of pollution-indicative DGGE bands and environmental conditions in the leachate plume from the Banisveld landfill. (A) Changes in the intensities of indicator bands (●, band 2; ○, band 5; ▲, band 8) (Fig. 3 and Table 2) relative to the total intensity of the track with distance along the flow path. (B) Changes in concentrations of aromatic micropollutants and DOC along the flow path (30). Average DOC (●) over three sampling events in 1998 to 1999, benzene (▲), and NTEX (naphthalene, toluene, ethylbenzene, and xylene) (○) concentrations, determined in June 1998, were used for the plot.

By contrast, bands 1 and 3 were confined to groundwater samples from the clean part of the aquifer, although they did not appear in all clean samples (Fig. 3 and Table 2). When the bands against redox process were analyzed (one group containing groundwater samples from iron-reducing parts of the aquifer and the other group containing groundwater samples from the nitrate-reducing parts of the aquifer), only two bands (bands 6 and 7) (Table 2 and Fig. 3) significantly correlated with the occurrence of nitrate reduction.

Phylogenetic analysis of *Geobacteraceae* communities. To obtain a more specific picture of the diversity of *Geobacteraceae* and of its relationship to hydrochemistry and biodegradation, phylogenetic analysis of cloned *Geobacteraceae* 16S rRNA genes was performed for five clone libraries, each of which was made for a composite groundwater sample that corresponded to a particular degree of pollution and redox condition (Table 1 and Fig. 1). Three clone libraries (Fe_P1, Fe_P2, and Fe_P3) corresponded to parts of the leachate plume where organic pollutants are being degraded (29, 30), while clone libraries Fe_C and NO_3_C are reference clone libraries corresponding to the clean aquifer with iron reduction and nitrate reduction as dominant redox processes, respectively. Thirty-three to 47 clones per clone library were categorized based on migration behavior in DGGE, after which one representative per DGGE type was sequenced. A number of non-*Geobacteraceae* sequences and chimeras between *Geobacteraceae* and non-*Geobacteraceae* were detected. Chimeras were especially observed in the three clone libraries derived from the polluted aquifer (11 out of 12 chimeras observed). The non-*Geobacteraceae* sequences were mainly found in the clone libraries corresponding to the clean aquifer (16 out of 19 cases). The percentage of correct *Geobacteraceae* clones in the three clone libraries from the polluted iron-reducing aquifer was 76 to 86% (Table 1). This percentage was nearly four times higher than that observed for the clone library corresponding to groundwater taken from the nitrate-reducing, clean aquifer above the plume (23%). The higher percentage of *Geobacteraceae* clones recovered from the iron-reducing part of the aquifer parallels a higher number of *Geobacteraceae*-specific DGGE types (Table 1).

Geobacteraceae sequences were subjected to phylogenetic analysis (Fig. 5). Most sequences from the three composite samples corresponding to polluted, iron-reducing parts of the aquifer grouped together. They were most closely related to clones (GenBank accession numbers AY013645, AY013647, and AY013648) that had been isolated previously from the same polluted aquifer (22) and are only distantly related to cultured *Geobacter* spp. (<97.3% similarity). Clones Fe_P1-3, Fe_P2-17, and Fe_P3-2 were highly identical (>99.1%) and showed a similar final migration position in DGGE, which corresponded to the dominant and distinctive band 2 in the DGGE profiles of groundwater *Geobacteraceae* (Fig. 3 and Table 2). Their DGGE positions were also similar to previously obtained clones (GenBank accession numbers AY013647 and AY013648), which corresponded to the dominant band in general *Bacteria* DGGE profiles (22). While most clones coming from the polluted aquifer were most closely related to these previously retrieved clones, none of the 13 different DGGE types from the clean parts of the aquifer was closely related to these clones.

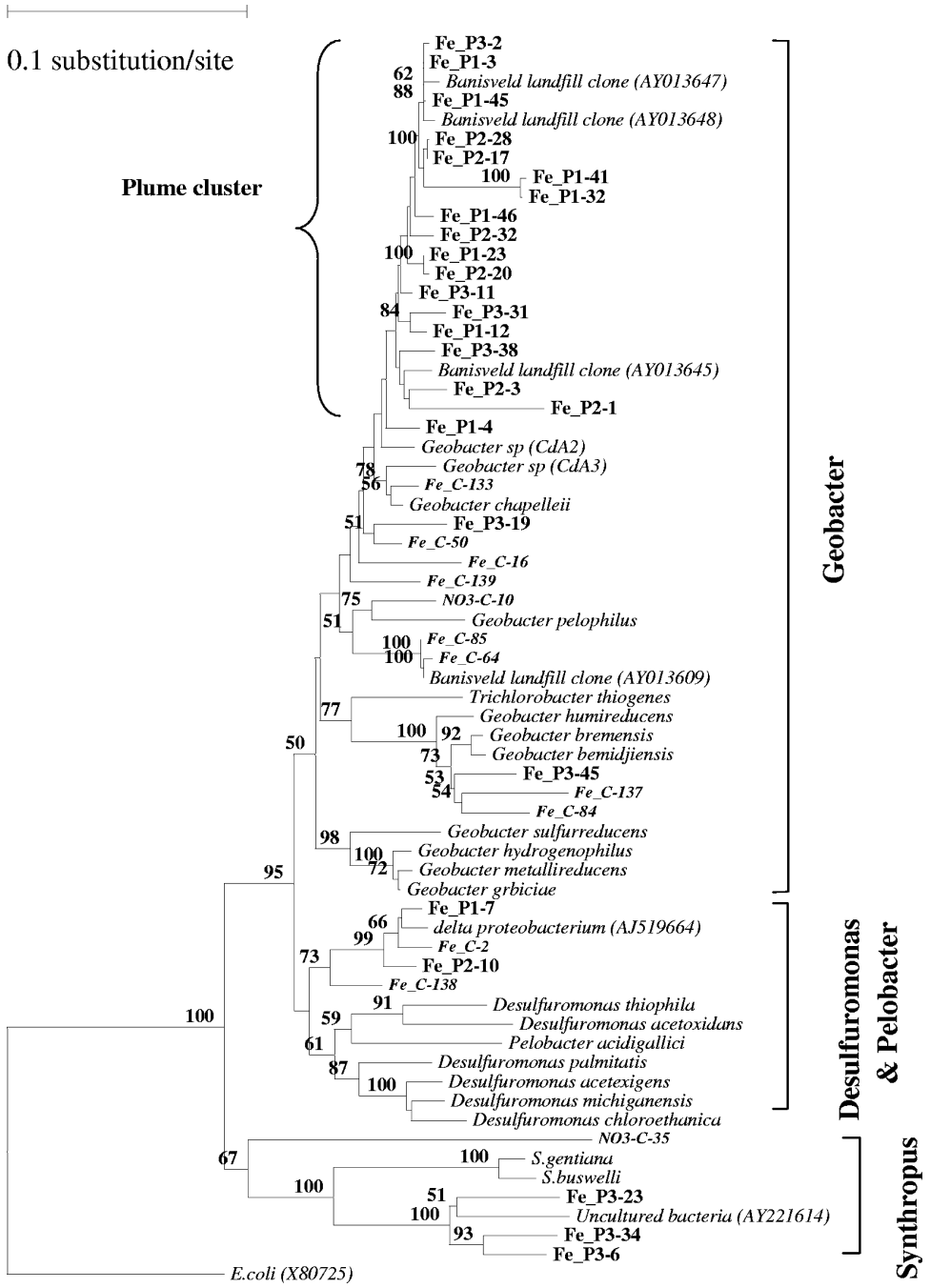

FIG. 5. Phylogenetic tree of *Geobacteraceae* clones from the Banisveld landfill leachate-polluted aquifer. A neighbor-joining analysis with the correction of Jukes and Cantor was performed on 737 unambiguous base positions. Only bootstrap values above 50% are shown. Codes of the clones correspond to the sampling location from which the clones were derived and are explained in Table 1; the last number is clone specific. All clones corresponding to the leachate plume are in boldface type, and all clones from the unpolluted aquifer are in boldface and italic type.

The phylogenetic tree further reveals that the clones from the clean parts of the aquifer were more diverse with respect to 16S rRNA gene sequence than clones from the polluted part of the aquifer. The majority of clones fell into the genus *Geobacter*, but clones Fe_P2-10, Fe_P2-7, NO_3_C-2, and NO_3_C-138 were most closely related to the genus *Desulfuromonas* of the *Geobacteraceae*.

DISCUSSION

Geobacteraceae are widely distributed in metal-reducing environments (2, 5–7, 10, 11, 22, 23, 25, 28) and are also associated with aromatic compound degradation (17, 23). However, detailed information about the relation between *Geobacter* community composition and environmental conditions is

scarce. Our results show that *Geobacteraceae* community composition in a landfill leachate-polluted aquifer corresponds to the level and type of pollution. They suggest that *Geobacteraceae* community composition may reflect biodegradation. A high *Geobacteraceae* diversity was observed, both outside and within the plume of pollution. Care has to be taken in the interpretation of DGGE data because of the fact that the applied primer set is not totally specific for *Geobacteraceae*.

Specificity of applied primers and implications of the methodology used for data interpretation. The applied *Geobacteraceae*-specific PCR primer set did not exclusively amplify *Geobacteraceae* rRNA genes. Especially when the dominant redox process was not iron reduction, a high number of non-*Geobacteraceae* sequences was observed. Previously, we observed that *Geobacteraceae* contributed to only a minor fraction (0.5%) of the microbial community in denitrifying groundwater (22). In the iron-reducing plume, *Geobacteraceae* contributed to 25% of the microbial community, based on both most-probable-number PCR and analysis of clone libraries constructed using *Bacteria*-specific primers (22). Therefore, the detection of high numbers of non-*Geobacteraceae* sequences seems to relate to the relative low abundance of *Geobacteracea*e in these samples. A second primer set for *Geobacteraceae*-specific PCR, amplifying *E. coli* 16S rRNA gene positions 494 to 825 (10), was employed to confirm the identity of *Geobacteraceae* clones, but it was found to amplify non-*Geobacteraceae* DNA fragments as well (data not shown). Other researchers have also reported the amplification of non-*Geobacteraceae* sequences when yet another *Geobacteraceae*-specific primer set was employed (7). After aligning 16S rRNA gene sequences from cultured members of *Geobacteraceae* and the sequences retrieved in this study, we were not able to design primers that target *Geobacteracaeae* more specifically (data not shown). In fact, primers *Geobacteraceae*-494F (10) and Geo564F (7), previously designed to detect *Geobacteraceae*, were found to contain a large number (>5) of mismatches and deletions towards more than 40% of the *Geobacteraceae* sequences retrieved in this study. We caution against concluding on *Geobacteraceae* diversity solely on the basis of the number of bands in *Geobacteraceae*-specific DGGE profiles, as this might well overestimate diversity due to the presence of bands that do not correspond to *Geobacteraceae*. DGGE data need to be complemented by phylogenetic analysis, as was done in this study.

Phylogenetic analysis revealed a cluster containing 20 closely related sequences derived from the polluted aquifer. Microheterogeneity within a sequence cluster has also been observed previously by others (9, 13, 27) and has also been observed for *Geobacter* sequences (25). Microvariation can be partially due to artifacts introduced by PCR and cloning (26). In our study, the 20 clones constituting the sequence cluster gave rise to 18 different final migration positions in DGGE that all fit with bands observed in the complex *Geobacteraceae* DGGE profiles directly generated from environmental samples (data not shown). A similar PCR-DGGE approach on DNA extracts from a single *Geobacter* strain and a mixture of two *Geobacter* species gave rise to 1 and 2 bands, respectively (data not shown), in agreement with what would be expected in the absence of PCR artifacts. Therefore, we conclude that PCR-induced artifacts were not a major factor contributing to the microdiversity in *Geobacter* sequences.

PCR-based 16S rRNA gene analyses are also prone to other pitfalls (33). However, since the same experimental approach was applied to all samples and PCR-DGGE results were well reproducible (data not shown), all samples should have suffered from the same pitfalls, allowing between-sample comparison of the DGGE profiles.

***Geobacteraceae* community structure in relation to pollution and biodegradation.** Previous work on the same DNA extracts employed in this study revealed that while cluster analysis of DGGE profiles obtained with general bacterial and archaeal primers discriminated between communities from polluted groundwater and clean water, it was not able to clearly distinguish between samples within the plume and to relate them to hydrochemistry and biodegradation (22). This study shows that community structures of the dominantly occurring *Geobacteraceae* are different within the plume: groundwater samples taken at 6 to 39 m from the landfill cluster together. They differ quite significantly from samples taken further downstream, which were more similar to the nearby nonpolluted groundwater. This is not surprising, since the aquifer close to the landfill has been exposed to leachate for the longest period of time and receives the highest concentrations of organic compounds as well as the most reactive organic matter.

The clustering correlates with the observed disappearance of the micropollutants ethylbenzene, xylene, and naphthalene over the first 39 m (29, 30). The biodegradation in the first part of the plume is also associated with high rates of iron reduction, presumably caused by the consumption of the more reactive DOC fraction there. This DOC (initial concentration, 3.1 mM) is 10-fold more reactive than a more persistent DOC fraction (6.1 mM) (29). The difference in iron oxide content in the polluted aquifer is unlikely to be an important factor that contributes to differences in the *Geobacteraceae* communities, as the content does not vary much along the flow path (30).

The differences in *Geobacteraceae* community structure in the plume were largely reflected by an intense band in the DGGE profiles corresponding to samples taken close to the landfill (<48 m). Statistical analysis showed that this band, as well as three other less intense DGGE bands, is indicative of groundwater pollution. The band was absent from most DGGE profiles of clean groundwater and present in all DGGE profiles of plume samples. The band corresponded to a previously encountered sequence that also gave rise to the most intense band in DGGE profiles generated with *Bacteria*-specific primers and corresponded to 23% of the clones in a *Bacteria*-specific clone library (22). Its apparent selection by pollution, its high intensity in DGGE profiles suggesting high abundance, and its correlation with high iron reduction rates suggest that the geobacters possessing this sequence play an important role in the attenuation of organic matter in the polluted aquifer.

Previously, Cummings et al. observed that metal contamination of Lake Coeur d'Alene (Idaho) sediments also selected for specific *Geobacteraceae* members (7). However, only six sediment cores were compared. As Cummings et al. discussed, their observations on *Geobacteraceae* distribution should be interpreted with caution, as many uncontrolled factors, such as different rivers functioning as the source of inoculum, local

heterogeneity, and organic content, may also have influenced the distribution of *Geobacteraceae*. In our study, we analyzed 27 different groundwater samples from a well-characterized aquifer and were able to identify *Geobacteraceae* sequences that are significantly indicative of environmental conditions.

***Geobacteraceae* diversity as a factor in resilience to pollution.** The *Geobacteraceae* present in the iron-reducing plume differ from those in clean groundwater, even though the clean aquifer underneath the landfill leachate plume is also iron reducing. A large diversity in *Geobacteraceae* was observed for the research location, especially in the clean, iron-reducing groundwater. Therefore, our analyses suggest that the geobacters encountered in the plume were selected by the pollution and have replaced the large pool of *Geobacter* species that had been present originally.

Other studies also revealed that species belonging to the *Geobacteraceae* can coexist. Cummings et al. (7) obtained four to nine different phylotypes for samples taken from a gradient of metal contaminants in Lake Coeur d'Alene. After in situ biostimulation of metal reduction in uranium-contaminated aquifer sediments, retrieved *Geobacteraceae* sequences could be grouped into two clusters (10). In benzene-oxidizing sediment from the petroleum-contaminated Bemidji aquifer (Minnesota), three phylotypes were enriched (23). After benzoate amendment of the Borden aquifer (Ontario, Canada), 11 closely related *Geobacter* sequences that differed from *Geobacteraceae* sequences retrieved from the unamended aquifer were obtained (25). Those studies and our data suggest that generally, species of the *Geobacteraceae* coexist and form a pool of functionally redundant (with respect to iron reduction) microorganisms. When environmental conditions change, such a pool may allow for a quick response.

A high diversity in *Geobacteraceae* in pristine aquifers might also be important for efficient biodegradation upon pollution: when more *Geobacter* spp. with different physiological abilities are present, a larger number of organic compounds might be degraded. However, 16S rRNA gene-based methods do not provide information on the physiology of *Geobacteraceae*. Enrichment, isolation, and physiological characterization are crucial for determining the functioning of *Geobacteraceae* in natural attenuation, for identifying the reasons behind the selection of particular *Geobacter* species upon organic pollution, and for determining the potential of *Geobacteraceae* to respond to changes in environmental conditions, such as the depletion of certain iron oxides. In contrast to many other environmentally dominant microorganisms, geobacters appear to be well amenable to cultivation (15, 25), and therefore, we are currently attempting to isolate the dominant *Geobacter* species from the Banisveld landfill aquifer.

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